

Enterococcus faecalis – the root canal survivor and ‘star’ in post-treatment disease

ISABELLE PORTENIER, TUOMOS M.T. WALTIMO & MARKUS HAAPASALO

In the past few years, *Enterococcus faecalis* has been the focus of increased interest both in medicine and dentistry. A recognized pathogen in post-treatment endodontic infections, *E. faecalis* is frequently isolated both in mixed flora and in monocultures. *E. faecalis* is probably the species that can best adapt to and tolerate the ecologically demanding conditions in the filled root canal. Eradication of *E. faecalis* from the root canal with chemomechanical preparation and using disinfecting irrigants and antibacterial dressings is difficult. In this article, the different factors that make *E. faecalis* a potential problem in medicine and dentistry are summarized, with special focus on the role of *E. faecalis* in post-treatment endodontic disease.

In the past few years, *Enterococcus faecalis* has been mentioned with increased frequency with regard to teeth with post-treatment disease (PTD), where it has also been detected as monocultures. The prominence of *E. faecalis* in root-filled teeth with apical periodontitis has made it a focus of attention as an etiological factor of PTD. It has been speculated that the presence of *E. faecalis* is encouraged by the conventional endodontic techniques, mainly the use of calcium hydroxide for root canal dressing. In order to appreciate fully the complexity of the microorganism, its involvement in PTD and possible ways to combat it, it is necessary to describe in detail the known background information about *E. faecalis*.

Enterococci are part of the normal flora in the oral cavity and gastrointestinal tract. They are recognized as potential human pathogens causing 12% of the nosocomial infections (1). Development of multiple resistances to various antibiotics in enterococci poses serious therapeutic problems. *E. faecalis* accounts for around 80% of all infections caused by enterococci (2, 3). Nosocomial and community-acquired infections caused by the genus *Enterococcus* include urinary tract infections, bacteremia, intra-abdominal infections and endocarditis (4–7). Enterococci are also frequently

isolated from mechanically ventilated (intubated) patients (8).

Enterococci pose increasing problems in medicine (acquired-nosocomial infections due to an increased resistance to various antibiotics), in food engineering and environmental control (*E. faecalis* is an indicator for fecal contamination in water and food) and in dentistry with therapy-resistant cases in endodontics. Therefore, there is a need to integrate knowledge from medical and dental studies on these organisms. This article summarizes the contemporary knowledge about *E. faecalis* and offers a brief description of different important aspects in medicine as well as in dentistry.

Enterococci

Until the mid-1980s, enterococci were not allocated to a separate genus, even though their unique characteristics were recognized among streptococci. The basic observations on staining, cell shape and arrangement as well as lack of catalase placed enterococci in the genus *Streptococcus*. With the serological Lancefield's classification and the discovery of the group D antigen, enterococci were classified as salt-tolerant group D

streptococci. However, the group D antigen is a lipoteichoic acid (LTA), one of the class of compounds that is found in virtually all Gram-positive bacteria and that is very different from the carbohydrate group antigen of other streptococci. This led to increased interest in the physiological characteristics and genetic relatedness of streptococci and other Gram-positive cocci. In 1984, enterococci were given a formal genus status after DNA–DNA and DNA–RNA hybridization studies demonstrated a more distant relationship with the streptococci, and two new genera, *Lactococcus* and *Enterococcus*, were introduced. The most important *Enterococcus* spp. are listed in Table 1.

Enterococcal cells are spherical or ovoid, occurring in pairs or short chains in liquid media (Figs 1 and 2). Endospores are not formed and some species can be motile by scanty flagella. They form creamy whitish colonies (Fig. 3), are Gram-positive, catalase-negative and able to grow in 6.5% NaCl, at temperatures ranging from 10°C to 45°C, and they can survive 30 min at 60°C and a pH over 9.6 (9). Most enterococci are facultative anaerobes, but some species are strict aerobes. A wide range of carbohydrates are fermented in glucose broth with the production mainly of lactic acid with a final pH of 4.2–4.6, sometimes with lower values (Fig. 4). Enterococci do not normally reduce nitrate and do not digest pectin or cellulose. They are ubiquitous and potentially pathogenic species that are able to acquire an increased resistance or phenotypic tolerance to many disinfectants or physical agents (10–

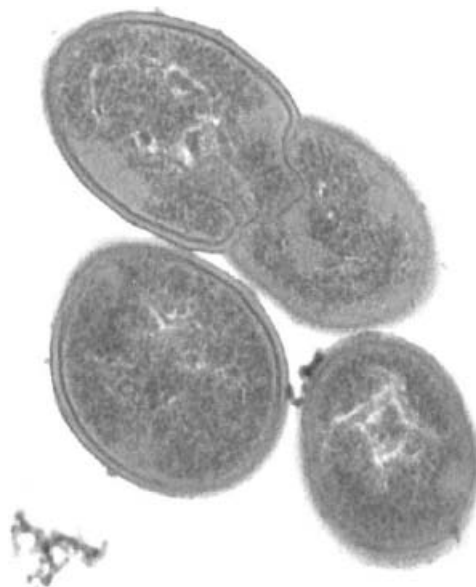


Fig. 1. A thin sectioned cell of *E. faecalis* (TEM, $\times 33\,000$).

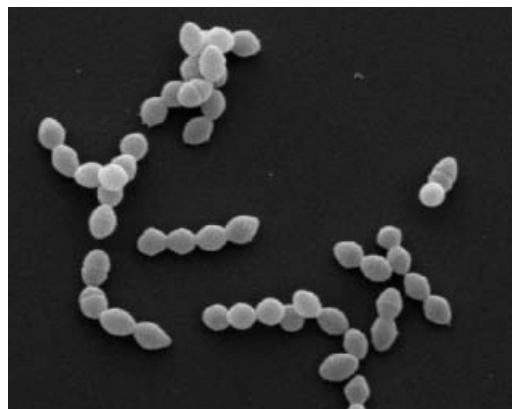


Fig. 2. A scanning electron micrograph of a dividing cell of *E. faecalis* ($\times 4000$).

Table 1. Most important *Enterococcus* species and their habitat

<i>E. faecalis</i>	Oral cavity, gastro-intestinal tract, animals, water, food
<i>E. faecium</i>	Oral cavity, gastro-intestinal tract, animals, water, food
<i>E. gallinarum</i>	Food, human (infrequently)
<i>E. casseliflavus</i>	Soil, plants, food, human (infrequently)
<i>E. avium</i>	Animal
<i>E. hirae</i>	Animal
<i>E. durans</i>	Human, animal, food

12). Growth on bile–esculin is a useful characteristic to identify enterococci (Fig. 5).

E. faecalis possess a group D carbohydrate cell wall antigen (Lancefield antigen), which is an intracellular glycerol teichoic acid associated with the cytoplasmic membrane. The cell wall contains a large amount of peptidoglycan and teichoic acid. The peptidoglycan (cross-linked peptide sugar), which is found in most of the bacterial cell walls, helps to maintain the microbe's shape and has a polysaccharide backbone of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acids (MurNAc). The chemical and structural analyses of the capsular polysaccharides have

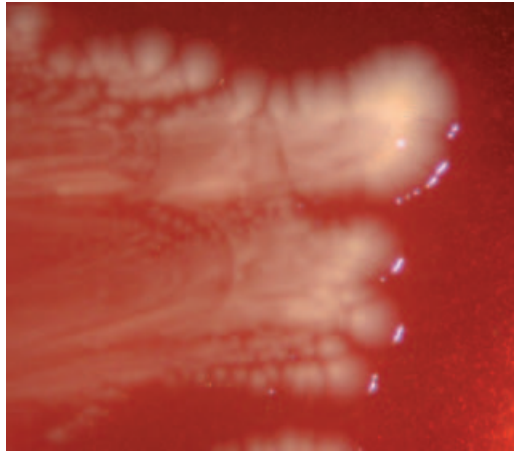


Fig. 3. Smooth, shiny colonies of *E. faecalis* on a horse blood agar plate.

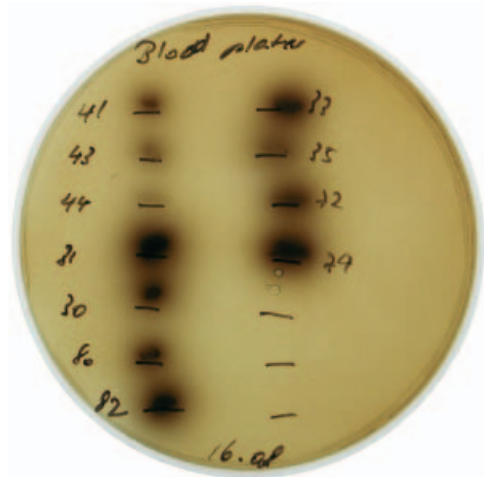


Fig. 5. *E. faecalis* and other enterococci grow on bile-esculin and stain the agar characteristically brown-black.



Fig. 4. Carbohydrate fermentation by *E. faecalis* (upper panel). Mannitol and sorbitol fermentation (yellow color) are among the characteristics that help to identify *E. faecalis* from other bacteria (lower panel).

shown glycerol teichoic acid-like molecules with a carbohydrate backbone structure and sialic acid. These polysaccharides are cross-linked with peptide bridges and contribute to the three-dimensional structure of peptidoglycan. Because of the location of the peptidoglycan on the outside of the cytoplasmic membrane and its specificity, the transglycosylation step has been indicated as a potential target for antibacterial medicaments.

Virulence and pathogenicity of enterococci

Virulence factors

Enterococci possess a number of virulence factors that permit adherence to host cells and extracellular matrix, facilitate tissue invasion, effect immunomodulation and cause toxin-mediated damage. These factors include: (1) aggregation substance (AS), (2) enterococcal surface proteins such as esp, (3) gelatinase, (4) a cytolysin toxin, (5) extracellular superoxide production, (6) capsular polysaccharides and (7) antibiotic resistance determinant.

(1) *AS*. The AS is a 37 kDa surface-localized protein, a plasmid-encoded adhesin. This adhesin mediates the cell-cell contact, which facilitates the plasmid exchange between recipient and donor strains (13–16). In this way, genetic material such as antibiotic resistance can be transferred between *E. faecalis* strains and other species (17). Aggregation substance may serve as a virulence determinant of *E. faecalis* in at least four distinct ways. (i) It plays a role in the dissemination of plasmid-encoded virulence factors, such as the enterococcal cytolysin and antibiotic resistance determinants, within the species. (ii) Further, it may facilitate the attachment of enterococci to renal and intestinal epithelial cells and colonization of these surfaces (18, 19). (iii) It may also protect against polymorphonuclear leukocyte (PMN) or macrophage-mediated killing by promoting phagocytosis of bacteria in a manner that

activates PMNs or macrophages, but does not result in microbial killing. The mechanism for this protection may be through a modification of phagosomal maturation (20–22). (iv) Finally, a study by Chow et al. (23) showed that aggregation substance and cytolysin have synergistic actions, which enhance the virulence by activating the quorum-sensing mode of cytolysin regulation. This will result in tissue damage and deeper tissue invasion.

- (2) *Surface protein esp*. Esp is a large chromosome-encoded surface protein with a particular architecture containing multiple repeat motifs. The role in virulence of esp is unclear, but it is speculated that the central repeat region may serve to retract the protein from the bacterial surface in order to hide the protein from the immune system, and that the N-terminal region of esp may participate in the interaction with the host. The esp-encoding gene (*esp*) has been sequenced in infection-derived *E. faecalis* strains and is shown to be often absent in less pathogenic species. This observation suggested a role for esp as a virulence factor in *E. faecalis* (24). Toledo-Arana et al. (25) demonstrated the relationship of the presence of the *esp* and the biofilm formation capacity in *E. faecalis*. They also showed that none of the *esp*-defective *E. faecalis* strains was able to form a biofilm, indicating a genetic association between the presence of *esp* and the presence of adhesins (25). It was also speculated that esp may have a direct role in the ligand-binding activity to extracellular matrix or an indirect influence in the modulation of ligand-binding activity of other molecules. Shankar et al. (24) suggested that the presence of esp could be responsible for the increased hydrophobicity and could therefore facilitate hydrophobic interactions. A recent study demonstrated that in the presence of the surface protein esp, hydrophobicity, biofilm formation and adherence to a biotic surfaces were increased (25).
- (3) *Gelatinase*. The main function of bacterial proteases is to provide peptide nutrients to the organisms. However, it is possible that proteases cause direct or indirect damage to the host tissues and then they can be classified as virulence factors. For *E. faecalis*, two secreted proteases have been described: gelatinase and a serine protease. Their secretion is autoregulated through the *ftr* system. Gelatinase is a non-plasmid-encoded metallo-endopeptidase, which is a strongly hydrophobic

protein and has a broad pH optimum between 6 and 8 (26). It can hydrolyze gelatin, casein, insulin, fibrinogen and small peptides (27). Mäkinen and Mäkinen (28) renamed this *E. faecalis* metallopeptidase as coccolysin (EC 3.4.24.30) because of its ability to inactivate human endothelin (a vasoactive peptide). However, the term gelatinase is commonly used in most studies. It has been reported that *E. faecalis* isolated from hospitalized patients has an increased production of gelatinase compared with community isolates (29, 30). In the study by Kanemitsu et al. (31), 45% of the *E. faecalis* hospital isolates produced gelatinase, whereas none of the *E. faecium* or *E. avium* isolates was gelatinase-positive. These findings corroborate the results by Elsner et al. (32), who found 55% of the *E. faecalis* strains to be gelatinase-producing, while no gelatinase production could be shown in the *E. faecium* isolates.

- (4) *Hemolysin*. Hemolysin (cytolysin), a plasmid-encoded toxin (33), is produced by beta-hemolytic *E. faecalis* isolates. It lyses erythrocytes, polymorphonuclear neutrophils and macrophages, kills bacterial cells and may lead to reduced phagocytosis (34–36). Based on amino acid analysis of the purified *E. faecalis* cytolysin, it has been classified as a type A lantibiotic. Lantibiotics are ribosomally synthesized peptides that contain the amino acid lanthionine and other modified amino acids that are normally not found in proteins. Other examples of type A lantibiotics are epidermin, nisin, subtilin and streptocin (37, 38). Further, they exhibit antibacterial activity (Fig. 6), forming pores in the cytoplasmic membrane of bacterial cells and energizing artificial phospholipid vesicles. In a study by Ike et al. (39), approximately 60% of the *E. faecalis* isolates derived from various sources were found to be hemolytic. They also showed that 85% of the hemolytic-positive strains exhibited aggregation, whereas only 49% of the non-hemolytic strains gave an aggregation response. Erythrocytes from different species have different levels of susceptibility to lysis by bacteria. While erythrocytes from rabbit, horse and human are sensitive, those from sheep or geese are partially or completely resistant (40). As sheep blood is often used in agar plates, this can lead to a misidentification of cytolysin-positive *E. faecalis* strains as non-hemolytic.

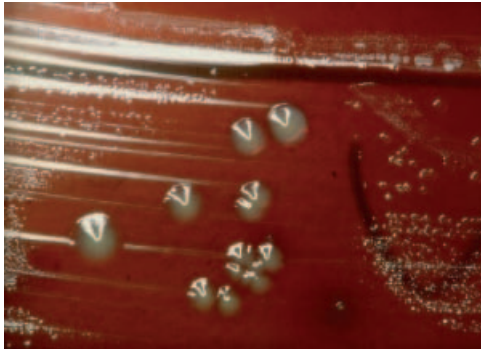


Fig. 6. *E. faecalis* (large colonies in the middle) produces substances that can inhibit the growth and even kill other bacteria.

- (5) *Extracellular superoxide*. Extracellular superoxide production is also associated with enterococcal virulence, and it has been shown that its production is significantly higher in invasive strains than in commensal isolates (41–43).

Modulation of the host immune response

PMNs are a critical component of the human host response against bacterial infections. Invading bacteria are opsonized by complement proteins or antibodies and subsequently phagocytosed and killed by PMNs. Studies investigating the potential immunological mechanisms associated with resistance to enterococcal infections conclude that neutrophilic killing of enterococci is mediated primarily by complement (44, 45), with antibodies playing a less important role. Bacterial cell lysis mediated by the membrane attack complex (MAC) may not play an important role in the immune response to enterococci because of the absence of the outer membrane in Gram-positive bacteria.

The bacteria are opsonized mainly by the third component of the complement (iC3b) and the phagocyte will bind these molecules through the CR3 receptor (46). The bacteria will be ingested, the PMN activated by formation of the phagosome and the bacteria will be killed and degraded through the respiratory burst. It has been shown that the enterococcal aggregation substance AS mediates opsonin-independent bacterial phagocytosis by human PMNs. However, the internalized AS-bearing enterococci are resistant to killing by macrophages through inhibition of the respiratory burst (21). AS-mediated resistance to killing is not limited to PMNs, but is also found with

other phagocytes, such as macrophages (21, 22). Studies have shown significant differences in the phagosomal maturation induced by the two mechanisms of binding (opsonin-dependent or opsonin-independent) to CR3 that may have contributed to the intracellular survival of AS-bearing *E. faecalis*. A significantly higher phagosomal pH has been reported after ingestion of non-opsonized AS-expressing *E. faecalis* than after ingestion of opsonized AS-negative *E. faecalis* (21).

A carbohydrate-containing moiety that is not sialic acid may be involved in the resistance (47). Bacterial capsules, largely polysaccharide, reduce hydrophobicity and attachment to phagocytes. On the other hand, aggregation substance increases the hydrophobicity of the enterococcal surface, which may induce localization of cholesterol to phagosome and prevent or delay fusion with lysosomal vesicles. Microorganisms that resist opsonization and, therefore, phagocytosis, use sialic acid, a common periodate-sensitive structure.

Membrane-associated lipoteichoic acids are polymers composed of a hydrophilic polyglycerolphosphate backbone linked via an ester bond to a hydrophilic glycolipid tail. Several biological characteristics of enterococcal lipoteichoic acid have been investigated. It has been shown that enterococcal lipoteichoic acid reversibly binds human erythrocytes as well as lipoteichoic acid from *Streptococcus pyogenes* (48). The acyl moiety of lipoteichoic acid is essential for binding. Lipoteichoic acid is continuously released from *S. pyogenes* (49), but it is not known whether enterococci also release lipoteichoic acid. As lipoteichoic acid bound by eucaryotic cells retains its antigenic specificity, this may be relevant to local inflammatory processes. When host cells with bounded LTA are exposed to plasma, they will suffer complement-mediated lysis (50). Lipoteichoic acid from clinically important Gram-positive organisms including enterococci also stimulates the production of interleukin-1, interleukin-6 and tumor necrosis factor α from cultured human monocytes (51). The levels of these monokines stimulated by enterococcal lipoteichoic acid were similar to those observed after exposure to Gram-negative lipopolysaccharides (51).

Antimicrobial resistance of enterococci

Most enterococci are intrinsically or naturally resistant to various antimicrobials including β -lactams

(cephalosporins and semisynthetic penicillinase-resistant penicillins), clindamycin, low concentrations of aminoglycosides and fluoroquinolones. They are naturally sensitive to ampicillin and vancomycin, but can acquire resistance to these antibiotics after exposure. They are able to develop resistance to tetracyclines, macrolides, glycopeptides (vancomycin and teicoplanin), chloramphenicol and to high concentrations of β -lactams as well as aminoglycosides. The acquisition of antibiotic resistance occurs either through the acquisition of resistance genes on plasmids or transposons from other organisms. Enterococci can secrete pheromones (16, 52), which are stimulating the synthesis of the surface aggregation substance (53, 54). This facilitates the contact between the cells and the formation of the mating aggregate, which finally will lead to the exchange of plasmids carrying resistance.

In the last few years, enterococci have received increasing attention because of the development of resistance to multiple antimicrobial drugs. This may be one explanation for its dominance in nosocomial infections. In addition to natural and acquired resistance to many agents, enterococci may also develop plasmid- and transposom-mediated resistance to tetracycline, erythromycin, high levels of trimethoprim and high levels of clindamycin. Vancomycin-resistant enterococci (VRE) probably represent the most serious challenge among many microbes with antibiotic resistance, as a source of human clinical infections in the past decades (Table 2). Two distinct phenotypes of transferable VRE have been described: the VanA phenotype, associated with a high level of inducible resistance to vancomycin and cross-resistance to teicoplanin; and the VanB phenotype, which usually has variable levels of inducible resistance only to vancomycin. The mechanism of acquired glycopeptide resistance in VanA and VanB enterococci is a cluster of genes encoding for a cell wall precursor that bind

vancomycin poorly (55, 56). Furthermore, the ability of enterococci to transfer plasmids to streptococci and staphylococci and the implications of a possible spread of penicillin- and vancomycin-resistance to these and other Gram-positive species are also of concern (17, 57, 58).

In Europe as well as in the USA, vancomycin-resistant *E. faecium* has caused most outbreaks of hospital infections and has also proved to be ampicillin-resistant (59). In cases of gentamycin-resistance, most of the isolates were VanB *E. faecium* isolates and they also were resistant to ampicillin and tetracycline (60). A study by Descheemaeker et al. (61) showed that the isolated gentamycin-resistant enterococci were VanA *E. faecium* (80%) and *E. faecalis* (15%).

Ecology

Stress response

Stationary phase (non-growth phase) is a common state of existence for microorganisms in nature. Different strategies have been developed by the bacteria in order to overcome low-nutrient (starvation) conditions. With increasing time of starvation the cell size will decrease, and in long-term starvation they will probably reach a minimum size (62–64). The rate of molecular synthesis is reduced in the transition from the growth to stationary phase (65–67). After 3–7 weeks of oligotrophic conditions, *E. faecalis* develops a rippled cell surface with irregular shapes and significant alterations, with collapsed envelopes detected in some cells (9). Contrary to growing cultures where cells are mainly organized in long chains, starved cells are organized as pairs while long chains can be seen only seldom (9).

Glucose-starved bacteria seem to be more resistant to different stresses (11, 68), suggesting that low-nutrient conditions induce the acquisition of a resistant phenotype in *E. faecalis*. For each type of stress, there exists a specific time required to reach the maximum resistance. Under low-nutrient conditions, the rate of protein synthesis will decrease; however the synthesis of 'starvation-induced proteins' will be important in protection of the cell against starvation (10, 68–73). Many recent studies have shown that the stationary phase enhances the protection of the cells against different stress challenges such as heat, H_2O_2 ,

Table 2. Incidence of vancomycin-resistant isolates in nosocomial infections

Europe	<3%
USA	13% (1995)
	26% (2000)

acidity, salt (NaCl), sodium hypochlorite (NaOCl), bile salts and sodium dodecyl sulfate (SDS) (11, 68–70, 72, 74).

Cell-mediated physiological changes of the organisms may be responsible for microbial resistance to disinfectants. Bacteria that are able to grow in a chemostat at low temperature and submaximal growth rates caused by nutrient limitation are resistant to several disinfectants (75, 76). Chlorine has disruptive effects on a variety of subcellular components and metabolic processes including inhibition of protein synthesis (77). If cells of *E. faecalis* in the growing phase are treated with low concentrations of hypochlorite, no increase of the resistance to high concentrations of NaOCl can be observed (68, 78). However, bacteria in a stationary phase (induced by glucose-starvation) showed a resistance against NaOCl 900-fold higher than that of a growing cell (72).

Glucose-starved cells of *E. faecalis* develop a cross-protection against heat, H₂O₂, acid (pH 3.7), ethanol (11) and NaOCl (68). Treatment of *E. faecalis* cells with either 0.08% bile salts or 0.01% SDS for 5 s was sufficient to enhance a significant resistance towards the respective detergent (10). A time of 30 min induced a nearly 100% survival of the cells. Further it was shown that cells pretreated with SDS (10) or heat (71) showed an increased cross-protection against bile salts and *vice versa*; an adaptation to bile salts enhanced cross-protection to SDS or heat. In contrast, no cross-protection against acid could be shown after adaptation to heat (71) or alkaline stress (72), and after an adaptation to bile salt the cells were even sensitized against acid treatment. No cross-protection between acid and bile salt could be shown; an acid challenge induced no increase in bile salts tolerance (71). However, cells that were alkaline-adapted developed a high resistance against bile salts (72). In 1998, Flahaut et al. (69) investigated the stress response in *E. faecalis* triggered by hydrogen peroxide (H₂O₂). They reported that pretreatment with a sublethal H₂O₂ concentration induced a 100-fold tolerance against the lethal effects of 45 mmol/L H₂O₂ challenge. Pretreatment with an acid (pH 4.8) enhanced the most efficient H₂O₂ cross-protection. NaCl (6.5%) and heat treatment (50°C) increased the tolerance to H₂O₂, whereas ethanol (69) and alkaline (69, 72) did not. Table 3 summarizes the main results from stress response studies with *E. faecalis*.

Table 3. Studies investigating the stress responses of cells of *Enterococcus faecalis*

Pretreatment	Stress	Changes in tolerance to stress	Reference
Glucose-starvation	Heat	Increased	11
	H ₂ O ₂		11
	Acid (pH 3.7)		11
	Ethanol		11
	NaOCl		68
0.08% bile	Respective detergent	Increased	10
0.01% SDS			10
0.1% SDS	0.08% bile	Increased	10
Heat			71
0.08% bile	0.01% SDS	Increased	10
	Heat	Increased	71
	Acid	Decreased	71
Heat	Acid	No change	71
Alkaline			72
Acid	Bile	No change	71
Alkaline	Bile	Increased	72
H ₂ O ₂	H ₂ O ₂	Increased	69
Acid (pH 4.8)			69
NaCl 6.5%			69
Heat (50°C)			69
Ethanol	H ₂ O ₂	No change	69
Alkaline			72
NaOCl	NaOCl	No change	68
			78

The viable but non-culturable (VBNC) state

After a long period of starvation, the number of culturable cells will decrease while the total number of bacteria remains at the initial level. An explanation is that the cells are dead and therefore not culturable. However, results of recent studies (79, 80) have indicated a new explanation: the cells have entered a state in which they are viable but non-culturable with standard microbiological techniques. To date, there are no adequate techniques to prove the viability of cells in that physiological state. As a consequence, resuscitation or restoration of cell division would support the VBNC hypothesis. It has been stated in several studies that changes in conditions such as temperature shift, or

addition of nutrients will lead to resuscitation of non-culturable bacteria (79–81). However, studies using mixed culture recovery indicate that resuscitation does not take place (82, 83). Experiments with two distinct strains showed that the non-culturable cells were dead and that the newly grown cells remained culturable (82). Moreover, the addition of nutrients did not lead to resuscitation (82). Whitesides & Oliver (84) showed that after a temperature upshift, non-culturable cells of *Vibrio vulnificus* could be resuscitated. An explanation for these results could be that the cells were passing through a state of injury before dying and were at the detection limit. By increasing the temperature they could recover from their injuries and grow. Repetition of the experiments by Bogosian et al. (83) led to the conclusion that the temperature shift had no effect on non-culturable cells.

Therefore, recent studies purporting to show that *E. faecalis* may reach the VBNC state and regain cell division (79, 80, 85) should be considered with caution, especially as the mixed culture recovery technique was not used.

In the future, techniques excluding the growth of remaining culturable cells have to be used in order to avoid misleading interpretation of the results. Further, it has to be clarified whether the non-culturable cells retain their infectivity. Bacteria that are able to enter the VBNC state are not detectable (at least not with the current microbiological techniques), and if they do retain their infectivity, they could present a risk to human health. In case they do not retain their infectivity, it should be clarified whether infectivity is regained after resuscitation.

***E. faecalis* and biofilm formation**

Many microorganisms are able to form surface-attached microbial communities, known as biofilms. Biofilms can be defined as communities of microorganisms attached to a surface and embedded in a matrix of polysaccharides and proteins forming a slimy layer. The matrix typically takes 85% of the volume of a biofilm. It is difficult to produce a biofilm in laboratories, because many laboratory-adapted strains have lost their ability to adhere to surfaces. Although bacteria forming a biofilm may not divide, they are viable and culturable once they are separated from the biofilm. An advantage of forming the biofilm-attached state is to accelerate the exchange of transmissible, genetic elements. It has been shown that there are increased rates of conjugation in bacterial

biofilms (86, 87). However, contrary to proposals that the attachment to a biofilm will result in a drastic change in the gene expression, Whiteley et al. (88) have shown differences in expression only for a few of the genes.

Biofilms can contaminate implants and catheters and are the source of 60% of hospital-acquired infections (89). A short time after the introduction of the plastic device into the body, host proteins and glycoaminoglycans are absorbed onto the surface of the biomaterial. Characteristics of the biomaterial as well as the composition of the host fluid will influence the absorption. The bacteria coming in contact with the device can bind to the proteins or glycoaminoglycans by receptor-specific or hydrophobic interactions. It has been shown that enterococci, like other Gram-positive microorganisms, are able to adhere and form biofilms on plastic surfaces (90). However, several factors responsible for the biofilm formation and its maintenance are unknown (89). Biofilm-based infections are rarely totally eliminated, even in individuals with a competent innate and adaptive immune response. Because of immune complexes and invading neutrophils, the tissues adjacent to the biofilm may undergo collateral damage (91). Antibiotic therapy may eliminate the symptoms and the bacteria that have been liberated from the biofilm, but it does not eradicate the cells that are embedded in the biofilm. Thus after the antibiotic therapy a recurrent infection may occur.

An immature biofilm of *E. faecalis* (12 h) on cellulose filters showed variation in the number of viable cells eluted from the biofilm, whereas a pseudo-steady state was developed and maintained from 12 to 96 h. During this time period, the number of cells attached to the biofilm and those shed to the perfusates was constant (92). In contrast, Lima et al. (93) found that 3-day-old biofilms lost more cells than the number of adhering cells. Anaerobic conditions or the presence of 5% CO₂ did not have an effect on the adhesion of enterococci to the microtiter polystyrene plates, with the exception of *E. hirae*. However, the presence of carbohydrates in the medium would strongly increase the biofilm formation of *E. faecalis* (94). *E. faecalis* had a greater ability to adhere to the microtiter polystyrene plates and form a biofilm than *E. faecium* (94). With maturation, biofilms on cellulose filters showed a decreased susceptibility to antibiotics and a reduced growth rate than planktonic cultures. Further, biofilms were resistant to vancomycin in a concentration of 4 × minimum inhibitory concentration (MIC) (92). They

were also less susceptible to teicoplanin (92). It has been suggested that different mechanisms of resistance to antibiotics are involved and that the resistance may not be caused by the presence of the glycocalyx. As the cells derived from the biofilm and regrown in planktonic cultures have the same MIC as the original cells, the resistance of the biofilm to glycopeptides is supposed to be related to the expression of a biofilm phenotype (92).

Lima et al. (93) tested the effect of different chlorhexidine- or antibiotic-containing medicaments on 1- or 3-day biofilms on cellulose nitrate membrane filters of *E. faecalis*. In the presence of clindamycin or clindamycin combined with metronidazole, the number of cells was reduced in the 1-day biofilm. Further, chlorhexidine-containing medicaments were able to reduce strongly the number of bacterial cells of *E. faecalis* in the 1- and 3-day biofilm. Spratt et al. (95) showed that 2.25% NaOCl was the most effective medicament on a 2-day-old *E. faecalis* biofilm, whereas 10% povidone iodine (Betadine) required 60 min to eliminate 100% of the cells, and in the presence of 0.2% chlorhexidine gluconate most of the cells survived after 60 min. However, the biofilms in Spratt et al.'s study, grown on cellulose nitrate membrane filters, were not standardized prior to testing, whereas Lima et al. (93) and Foley & Gilbert (92) related their results to the initial number of adhering cells.

The first hypothesis for the resistance of bacterial biofilms to antibiotics is the reduced or slow penetration of the medicament through the biofilm. Studies have shown that antibiotics can penetrate quickly through the biofilm matrix (96, 97). However, the antibiotic may be deactivated in the biofilm. Biofilm of wild-strain *Klebsiella pneumoniae* deactivated ampicillin in the surface layer, whereas a biofilm of β -lactamase-negative strains of the same bacterium did not (98). Another reason for a slower penetration could be absorption of the antibiotics into the biofilm matrix (99–101). A study by De Beer et al. (102) showed limited penetration of chlorine into the biofilm matrix.

The second hypothesis is based on possible changes in the chemical environment in the biofilm. Xu et al. (103) have shown that oxygen can be completely consumed, leading to anaerobic conditions in the depth of the biofilm. Some antibiotics require aerobic conditions in order to be effective; therefore, bacteria in deeper layers in a biofilm with anaerobic conditions will be protected. Tack & Sabath (104) showed that aminoglycosides are clearly less effective in anaerobic

than in aerobic conditions. Accumulated acidic waste products can also lead to a difference in pH and thus have an antagonizing effect on the antibiotics. It has also been suggested that the depletion of a substrate or accumulation of waste products could result in bacteria entering a non-growing state. This would protect the bacteria from being killed by penicillin as it targets cell-wall synthesis and therefore kills only growing cells (105). Further, changes in the osmotic conditions within the biofilm could result in osmotic stress responses (106). These responses could lead to changes in the relative proportion of porins, so that the cell membrane permeability to antibiotics is reduced.

The third hypothesis is still speculative and suggests that a subpopulation of bacteria in a biofilm forms a phenotypic state. In this state, the bacteria are highly protected and the cell differentiation is similar to spore formation. This hypothesis is supported by recent studies indicating that in immature or newly formed biofilms, the susceptibility to antibiotics is dramatically decreased even though the formed layer is too thin to pose a barrier to penetration to either medication or metabolic substrates (92, 93, 95, 107, 108). In summary, it is clear that all the mechanisms depend on the multicellular nature of the biofilm, which explains also why bacteria that are shed from a biofilm revert rapidly to a susceptible phenotype.

Adherence to extracellular proteins

Adherence of bacteria to host tissues is an important step in the invasion of the tissue and the establishment of an infection. Bacteria can adhere through specific adhesin-ligands to the host extracellular matrix. The extracellular matrix (ECM) is composed of glycoaminoglycans (heparin, heparan sulfate, chondroitin sulfate) and glycoproteins such as collagen, fibronectin, lactoferrin, laminin, vitronectin and albumin. Collagen is the predominant tissue protein; albumin is present in high concentrations in serum; and fibronectin is found in soluble form in blood plasma and in a less soluble form in the connective tissue and the basement membranes of mammalian cells. In cases of endocarditis, the surface of damaged heart endothelial tissue is exposed, and bacteria that have a specific binding site for fibronectin are able to bind (109).

Results related to *E. faecalis* binding fibronectin are contradictory. Shorrock & Lambert (110) reported that *E. faecalis* bind to fibronectin to a significant level,

whereas more recent studies have reported a low level of fibronectin binding by *E. faecalis* (111, 112). Xiao et al. (111) reported that at 37°C *E. faecalis* showed little adherence to immobilized laminin, collagen types I and IV. The adherence was increased when the experiments were performed at 46°C. No increase in adherence was reported for fibronectin and fibrinogen at 46°C. As the adherence was found to be reduced after proteolytic digestion of the proteins (111, 112), and heat treatment (110–112), it was suggested that protein-binding components are involved in the process of adherence. However, a study by Rozdzinski et al. (113) showed that in the presence of aggregation substance, enterococcal adherence to fibronectin was increased. In addition, Shorrock & Lambert (110) showed that the binding of *E. faecalis* to fibronectin or albumin does not appear to involve lipoteichoic acid.

Differences in the results of these studies may be explained by various factors such as use of different strains, differences in growth conditions and differences in the experimental protocols. Further, some studies have analyzed the binding of enterococci to ECM proteins, whereas others have analyzed the opposite, which make comparisons of results from different studies difficult.

Non-oral infections caused by enterococci

Enterococcus faecalis is responsible for about 80% of all infections caused by enterococci, with *E. faecium* responsible for the remaining 20% of the infections (2, 3). In a survey of 15 000 isolates, only 2% of *E. faecalis* strains were resistant to ampicillin or vancomycin compared with 83% of *E. faecium* strains. However, even though *E. faecalis* is less resistant to antibiotics, it is still responsible for the major part of infections. These observations indicate the existence of additional virulence factors that enhance the virulence of *E. faecalis*. Enterococci are responsible for 8–15% of infective endocarditis (114–117), and have a high affinity for heart valve tissue like streptococci and staphylococci (118–120).

Urinary tract infections

Enterococci caused 5–15% of the nosocomial urinary tract infections as reported in USA (121–123). One study showed that the most common pathogen isolated

in urinary tract infection was *Escherichia coli* and accounted for 47% of the infections (124). VRE could be recovered in studies in USA and accounted for around 5–10% of the urinary tract infections caused by enterococci, whereas in Canada enterococci were not commonly isolated (123, 124). As urinary tract infections caused by enterococci are most likely to be acquired in hospital or long-term care settings, they will be at a higher risk of multiple resistances against antibiotics (125–127).

Bacteremia

In the 1980s, enterococci were the sixth most common cause of nosocomial bacteremia. This incidence has since increased dramatically; to date, enterococci are the third leading cause for nosocomial bacteremia (128). Up to two-thirds of the isolates are *E. faecalis*, approximately one-third *E. faecium* (129, 130). Some 35% of the enterococcal bacteremia isolates were resistant to vancomycin (129). The portal of entry for enterococcal bacteremia was a genitourinary, an intra-abdominal or an intravascular catheter (129, 131, 132). The most common site of infections among patients with bacteremia caused by vancomycin-resistant enterococci was vascular catheters (129).

As the treatment of endocarditis is more difficult than that of a bacteremia caused by a specific, non-cardiac source, it is important to know that the bacteremia is not secondary to endocarditis. It was shown that patients with hospital-acquired enterococcal bacteremia are prone to develop infectious endocarditis (130); however, Maki & Agger (133) found no correlation between hospital-acquired bacteremia and endocarditis. It has been reported that even though the source was known, the overall mortality rate from enterococcal bacteremia was around 19% (129, 134). Noskin et al. (134) reported that bacteremias caused by *E. faecium* had a higher mortality rate than those caused by *E. faecalis* (50% vs. 11%).

Intra-abdominal infections

Enterococci can be frequently isolated from intra-abdominal infections as well as pelvic and soft-tissue infections of this region. They rarely present as monoinfections in these sites but are more commonly isolated from a mixed microbial flora.

It is debated whether these organisms do play an important role in wound infections, as it is not clear

whether the tissue damage or abscess formation is caused by enterococci or by the other microorganisms present.

Endocarditis

Enterococcal endocarditis is a serious condition, because it is difficult to treat even though the enterococci causing the endocarditis are susceptible to antibiotics. Enterococci exhibit a low-level, intrinsic resistance to many antibiotics (e.g. β -lactams, aminoglycosides, clindamycin and lincomycin and fluoroquinolones and glycopeptides). The severity of infection caused by enterococci is increased because of the acquisition of high-level resistance and multidrug resistance. For an effective therapy, two drugs that have a synergistic effect are often necessary (135–137). In case of VRE or high-level aminoglycoside-resistant enterococci, the antibiotic often fails to eradicate the source, relapses occur and surgery is necessary in order to remove the infected valve (138).

Eight to 15% of endocarditis cases are caused by enterococci (114–117), and this rate has not increased during the last few decades. *E. faecalis* is more often responsible for endocarditis than *E. faecium* (130). It is known that enterococcal endocarditis may cause serious and life-threatening conditions. The mortality rate for enterococcal endocarditis is around 20% and similar to the mortality for staphylococcal endocarditis (138–140).

Oral enterococcal infections

Enterococci in the normal flora of the oral cavity

Only a few studies have focused on the occurrence of enterococci in the oral cavity. Enterococci have been isolated in small numbers from the oral cavity of a number of people (141). *E. faecalis* is the most commonly isolated species of enterococci. According to Jett et al. (142), enterococci are commensal organisms well suited for survival in intestinal and vaginal tracts and the oral cavity (142). In an early report, Williams et al. (143) found enterococci in the saliva of 21.8% of 206 investigated persons. Smyth et al. (144) studied the carriage rates of enterococci in the dental plaque of hemodialysis patients and in different control groups. The overall carriage rates of enterococci of the University group, the toothache patient

group, the hemodialysis patients and the dialysis unit staff did not differ significantly from each other, ranging from 5% to 20%. According to the results, age, sex, history of recent antibiotic therapy and elapsed time since the last dental visit did not significantly affect isolation rates. The *Enterococcus* most commonly isolated from subjects was *E. faecalis*, followed by *E. liquefaciens*. Only one subject harbored *E. durans* in dental plaque. Ten of the 21 subjects yielding enterococci harbored two different enterococci in their plaque. The isolation of *E. liquefaciens* alone or in combination with *E. faecalis* did not correlate with subject–history parameters (144). Recently, Sedgley et al. (145) investigated the prevalence, phenotype and genotype of oral enterococci. Enterococci were detected in oral rinse samples from 11% of 100 patients receiving endodontic treatment and 1% of 100 dental students with no history of endodontic treatment. All enterococcal isolates were identified as *E. faecalis*.

Almstahl et al. (146) studied the oral microflora of patients with primary Sjogren's syndrome. The patients displayed an increased frequency of *C. albicans*, *Staphylococcus aureus*, enterics and enterococci on the soft mucosa and tongue. *E. avium* and *E. faecalis* have been reported in the subgingival microflora in patients with acquired immunodeficiency syndrome (147). Bergmann (148) studied changes in the aerobic and facultatively anaerobic oral microflora during remission–induction chemotherapy in patients with acute myeloid leukemia. The material included 10 patients who were studied during a period of 28 days. No changes in the relative proportion of individual microorganisms or acquisition of new microorganisms occurred during the antineoplastic treatment. During antibacterial treatment, which followed the antineoplastic treatment in all patients, a 100-fold decline occurred in the median salivary concentration of microorganisms within the first 7 days. During this period, members of the normal flora became undetectable in five patients, and Enterobacteriaceae, *E. faecalis* or *Candida* spp. became parts of the quantitatively predominant oral microflora in seven patients. After the termination of the antibacterial treatment, the concentration of the microorganisms increased to their original level and normal flora became re-established within 8 days.

E. faecalis is clearly a part of the human oral flora. However, in healthy individuals not being treated with wide-spectrum antibiotics, the relative amount of

enterococci is quite small, often below the detection level when normal sampling and culture methods are used. The dominance of *E. faecalis* in infected, filled root canals, however, is an indication that its occurrence in the oral cavity may be higher than suggested by most studies.

Marginal periodontitis

Enterococci are not typical members of the flora in periodontal infections. However, several studies have reported isolation of enterococci also in periodontal infections. Colombo et al. (149) measured serum antibody levels of selected bacteria in patients with periodontal disease. *E. faecalis* was among those bacteria that elicited high serum antibody, both in the successfully treated and in refractory patients. In another study, Colombo et al. (150) investigated the clinical and microbiological features of refractory periodontitis. Sequencing of the isolated bacteria revealed the presence of several non-typical bacteria, including *E. faecalis*. A study of the subgingival microbiota of Brazilian subjects with untreated chronic periodontitis also documented *E. faecalis*, which was detected significantly more often and/or in higher levels in the periodontitis group than in control subjects (151). Rams et al. (152) have studied the prevalence of enterococci in human periodontitis. Subgingival enterococci occurred in 1% of early-onset periodontitis patients and in approximately 5% of adult periodontitis patients. In this study, *E. faecalis* was the only enterococcal species recovered, and all but one isolate belonged to the same biotype.

The role of enterococci in the pathogenesis of periodontal diseases remains unclear. It cannot be excluded that its increased occurrence in diseased sites and in superinfections is an indication of ecological selection rather than evidence of its important role in pathogenesis.

Enterococci in primary apical periodontitis

A strong dominance of strictly anaerobic bacteria is typical of primary apical periodontitis, together with some facultative anaerobic bacteria such as streptococci, lactobacillus and actinomyces. Primary apical periodontitis has a wide variety of bacterial combinations; usually three to six species can be isolated from one tooth using conventional culturing methods (153–

161). In a recent study, using both culturing and polymerase chain reaction (PCR) methods, Munson et al. (162) reported a higher number and a greater diversity of species in the necrotic root canal than found previously. This can be explained by the high proportion of strains, which are difficult to culture even with modern anaerobic techniques. However, the role of uncultivable species in apical periodontitis is still unknown. The number of species has been shown to be higher in teeth with large periapical lesions than in teeth with minor lesions (156).

Anaerobic bacterial flora in primary apical periodontitis is partly a consequence of the ecological conditions in the necrotic root canal: low redox potential and nutrients rich in peptides and low in carbohydrates. Studies on animals, using experimental root canal infections with a known mixture of bacteria, have also demonstrated a strong shift in the infective flora towards obligate anaerobic species (163).

Enterococcus spp. are not typical isolates in primary apical periodontitis, when sampling is performed at the beginning before any treatment procedure. In most, if not all, studies where the initial diagnosis of primary apical periodontitis has been well documented, enterococci are not found (153–161). In many studies where enterococci have been found, the pretreatment diagnosis has not been given in detail, or the source of specific bacterial isolates has not been indicated when both primary and secondary infections have been studied. Therefore, to some extent it has been uncertain whether or not *E. faecalis* can be found in untreated teeth with apical periodontitis. However, already 40 years ago, Engström (164) reported enterococci in 12.1% of culture-positive teeth at the beginning of primary treatment of necrotic root canals. Recently, Siqueira et al. (165) analyzed the prevalence of *Actinomyces* spp., streptococci and *E. faecalis* in primary root canal infections by using a molecular genetic method. Samples were obtained from 53 infected teeth, of which 27 had acute periradicular abscesses. The occurrence of 13 bacterial species was studied by using whole genomic DNA probes and checkerboard DNA–DNA hybridization. All root canal samples contained bacteria as demonstrated by PCR. The checkerboard DNA–DNA hybridization assay allowed the detection of streptococci in 22.6% of the samples, *Actinomyces* spp. in 9.4%, and *E. faecalis* in 7.5%. In asymptomatic lesions the most prevalent species were *S. intermedius* (11.5% of the cases), *E.*

faecalis (11.5%) and *S. anginosus* (7.7%). The occurrence of *E. faecalis* in acute infections was clearly lower than in asymptomatic teeth. *S. constellatus* was the only species positively associated with acute periradicular abscess ($P < 0.01$).

Enterococci in the root canal after initiation of treatment

There are no data on the occurrence of enterococci in the root canal after beginning treatment of teeth with the verified diagnosis of primary apical periodontitis. However, in 1975, Mejare (166) examined the bacteriological status of 612 root canals at the time of filling. The samples were obtained by dental students during a continuous period of 1 year. Twenty-nine isolates from 27 (29.3%) of the 92 positive cultures were identified as enterococci. Biochemical tests classified the isolated enterococci into the following taxa: *S. faecalis* subsp. *faecalis* (10), *S. faecalis* subsp. *zymogenes* (3), *S. faecalis* subsp. *liquefaciens* (8), and atypical variants of *S. faecalis* (6), *S. faecium* var. *faecium* (1) and *S. faecium* var. *durans* (1). According to the author, the enterococci are of special interest in studies on the influence of infection at the time of root filling on the prognosis of root canal therapy.

Sirén et al. (167) studied the correlation between several clinical parameters and the occurrence of enterococci in teeth where treatment did not result in healing. The clinical treatment history of 40 *Enterococcus*-positive teeth and 40 *Enterococcus*-negative teeth was compared in order to explain the occurrence of enterococci in some teeth but not in others. The results showed that the prevalence of isolating *E. faecalis* in the root canal increased significantly if the canal had been left unsealed between appointments and, in particular, when appointments were many. The obvious conclusion from this study is that compromised asepsis during endodontic treatment is an important causative factor for contamination of the root canal by *E. faecalis*.

Post-treatment apical periodontitis and retreatment

It has been clearly established that enterococci are the dominant bacteria in retreated teeth with post-treatment apical periodontitis. Contrary to primary apical periodontitis, anaerobic bacteria constitute the minority of the flora, and are isolated less frequently.

E. faecalis is the most frequently isolated species and is usually the predominant isolate in the canal. In a classical study, Engström (164) investigated the occurrence of enterococci in 223 teeth. Growth was found in 134 samples (60.1%), and enterococci were found in 20 cases (14.9%). The frequency of isolation of enterococci was 12.1% (of culture-positive cases) for primary treatments and 20.9% for previously root-filled teeth. Molander et al. (168) retreated 100 root-filled teeth with apical periodontitis, and found bacteria in 68% of the teeth. *E. faecalis* was the most frequent isolate, found in 47% of the culture-positive teeth. In the same study, 20 root-filled teeth without disease were similarly cultured for microbial presence (168). Mostly sparse growth was detected in thirteen teeth, while no bacteria were found in seven teeth. *Enterococcus* sp. was found in only one tooth in this group (8% of culture-positive teeth). Sundqvist et al. (169) retreated 54 teeth with post-treatment disease. They found microbial growth in 24 teeth (45%), while 30 teeth gave no growth. *E. faecalis* was found in nine teeth (38% of the culture-positive teeth) and was the most frequently isolated species. On each occasion, *E. faecalis* was isolated in pure culture. Hancock et al. (170) retreated 54 root-filled teeth with post-treatment disease and obtained microbial growth from the root canals of 33 teeth (61%). They found *E. faecalis* in 10 of the culture-positive teeth (30%); in six teeth, *E. faecalis* was present in pure culture.

Peciuliene et al. (171) retreated 40 root-filled teeth with asymptomatic apical periodontitis. Microbial growth was detected in 33 teeth (83%), and *E. faecalis* was isolated in 21 teeth (64% of the culture-positive teeth). In 11 teeth, *E. faecalis* was the only isolate, and in 10 teeth it was isolated together with other bacteria or yeast. In eight of 10 teeth where *E. faecalis* was found in a mixed infection, it was the dominant species. The size of the lesion was correlated with the microbiological findings, revealing an average lesion diameter of 6.8 mm for *E. faecalis* mixed infections, 5.7 mm for *E. faecalis* pure infections and 4.3 mm for mixed infections without *E. faecalis*. Interestingly, the average lesion size in the 7 teeth where no bacteria could be cultured was also 5.7 mm. Total colony-forming unit (cfu) counts (total number of microbes from the sample) in this study were between 40 and 7×10^7 cfu, without correlation with the size of the apical lesions (171). It is obvious that in root-filled teeth, the localization of the bacteria in the root canal

greatly depends on the space available, and the root filling may limit the possibilities of the infective flora to interact with the periapical tissues through the apical foramen. Pinheiro et al. (172) retreated 60 root-filled teeth with post-treatment disease, and found that 51 teeth (85%) were culture-positive. *E. faecalis* was by far the most frequent isolate, present in 45% of all teeth and 53% of the culture-positive ones. In addition, *E. faecium* was found in one tooth. Eighteen of the 27 *E. faecalis* strains were isolated in pure culture (172).

Recently, Siqueira & Rocas (173) analyzed microorganisms associated with post-treatment disease using PCR. Root canal samples were taken from twenty-two root-filled teeth with persistent disease, which were selected for endodontic retreatment. DNA was extracted from the samples and analyzed for the presence of 19 different species by using the PCR. All samples were positive for at least one of 19 species studied. *Enterococcus faecalis* was the most prevalent species and was detected in 77% of the teeth. Interestingly, other prevalent species were *Pseudoramibacter alactolyticus* (52%), *Propionibacterium propionicum* (52%), *Dialister pneumosintes* (48%) and *Filifactor alocis* (48%). *Candida albicans* was found in 9% of the samples. The mean number of species in canals filled 0 to 2 mm short of the radiographic apex was 3 (range, 1–5), whereas canals filled shorter than 2 mm from the apex yielded a mean of 5 species (range, 2–11). This difference was statistically significant ($P < 0.05$).

The role of enterococci in acute endodontic infections and flare-ups has not been studied much. The study of Pinheiro et al. (172) indicated that it is primarily the anaerobic bacteria and not *E. faecalis* or other enterococci that are associated with acute symptoms in teeth with post-treatment disease. In a study of primary apical periodontitis using PCR for bacterial detection, Siqueira et al. (165) found *E. faecalis* more often in symptom-free teeth than in teeth with acute symptoms. Although *E. faecalis* is the dominant species in root-filled teeth with apical periodontitis that resisted many treatment procedures, there is no evidence that it is responsible for severe acute infections.

Susceptibility of *E. faecalis* to interappointment dressings and irrigants

A variety of antimicrobial agents have been tested for their ability to eliminate *E. faecalis* from the root canal

system. These include both interappointment dressings, such as calcium hydroxide, camphorated para-monochlorophenol, camphorated phenol and mixed antibiotic–steroid combinations, as well as irrigants such as NaOCl, chlorhexidine digluconate, chlorhexidine acetate and iodine compounds (174–182). *E. faecalis* is the most resistant bacterium against calcium hydroxide, both *in vivo* and *in vitro* (174). Only oral *Candida* species are generally more resistant than *E. faecalis* to direct exposure to saturated calcium hydroxide solution (183). *In vitro* studies show that *E. faecalis* is killed within 6–10 min in saturated calcium hydroxide (183). However, clinical experience and *in vitro* experiments using dentine blocks inoculated with *E. faecalis* have clearly shown that it is difficult, if not impossible, to kill *E. faecalis* in dentine, even after prolonged periods of incubation with calcium hydroxide (176–178).

NaOCl is effective against *E. faecalis* both in buffered and unbuffered solutions (184). However, a recent study by Gomes et al. (185) suggested that a 30-min incubation is required to eradicate *E. faecalis* completely with 0.5% NaOCl. This seemingly contradictory result with other studies may be explained by the fact that different detection levels have been used in different studies. The results of Gomes et al. (185) may partly explain the persistence of *E. faecalis* in the root canal. Peciuliene et al. (171) studied the effect of instrumentation and antibacterial irrigation on *E. faecalis* in retreated teeth *in vivo*. Root fillings were removed with endodontic hand instruments and chloroform was not used to avoid a negative effect on microbial viability as suggested by Molander et al. (168). After the first microbiological sample, the canal was cleaned and shaped with reamers and Hedstroem files, using 2.5% NaOCl (10 mL) and 17% neutral EDTA (5 mL) as irrigating solutions. All canals were prepared to size #40 or larger, 1 mm short of the radiographic apex. Chemomechanical instrumentation was completed in one appointment in all teeth. The canals were dried with paper points and a second microbiological sample was taken from all teeth. The second sample revealed *E. faecalis* in six of 21 teeth (29%) where it was found in the first sample (171). In the same study, none of the six *C. albicans* strains that were present in the first sample could be found in the second sample after preparation with NaOCl and EDTA irrigation. The cfu counts (number of bacteria) in the second samples were always below 1% of the cfu

counts in the first sample. These results clearly demonstrate that sodium hypochlorite irrigation cannot predictably eliminate *E. faecalis* from the root canal.

Recently, a new root canal irrigating solution, MTAD, which is a mixture of a tetracycline isomer, an acid and a detergent, was introduced (186, 187). MTAD is to be used either alone or in combination with other irrigating solutions such as NaOCl. MTAD effectively removes the smear layer and has antibacterial activity (186, 187). Experiments using infected dentine surfaces or infected dentine have demonstrated that MTAD effectively kills salivary bacteria even after 5 min of incubation, and has a potential to facilitate eradication of *E. faecalis* from infected dentine when used together with NaOCl (188, 189).

Chlorhexidine is bactericidal in clinically adequate concentrations. It is effective against a wide range of both Gram-negative and Gram-positive bacteria as well as against yeasts (180, 182, 190–193). Gomes et al. (185) demonstrated *in vitro* that only 30 s were required to eradicate *E. faecalis* completely by 0.2–2% chlorhexidine gluconate solutions in water, compared with 5 min or longer with NaOCl in concentrations below 5.25%. Gomes et al. (185) also showed that while 0.2% chlorhexidine liquid killed *E. faecalis* in 30 s, chlorhexidine gel with the same concentration required 2 h to achieve the same result. Heling et al. (180) demonstrated the superiority of a chlorhexidine-containing sustained-release device to calcium hydroxide in killing *E. faecalis* in inoculated dentine blocks. Chlorhexidine significantly reduced the bacterial population in the initially inoculated groups and prevented secondary infection of the dentinal tubules in the reinoculated group. By contrast, calcium hydroxide did not show any antibacterial activity and failed to disinfect the dentinal tubules or prevent growth after a reinoculation period. Lenet et al. (194) showed a better antibacterial effect of chlorhexidine gel than calcium hydroxide in eliminating *E. faecalis* from inoculated dentine *in vitro*. The antibacterial efficacy of 2% chlorhexidine solution against *E. faecalis* in inoculated dentine was further shown in human teeth specimens by Basrani et al. (195).

Lui et al. (196) compared the antibacterial effect of calcium hydroxide paste and chlorhexidine-impregnated gutta-percha points against *E. faecalis* in human maxillary premolar roots *in vitro*. Both medicaments showed better antibacterial activity than negative

control; calcium hydroxide was, however, more effective than the chlorhexidine-impregnated gutta-percha points.

Sukawat & Srisuwan (197) compared the effect of pure calcium hydroxide and calcium hydroxide combined either with chlorhexidine (0.2%) or camphorated monoparachlorophenol (CMCP) using the human dentine block model and *E. faecalis* as a test organism. Calcium hydroxide combined with CMCP effectively killed the test organism in dentine, while no difference was found between pure calcium hydroxide and calcium hydroxide combined with chlorhexidine. However, experiments using higher concentrations of chlorhexidine appear to have different results. Evans et al. (198) showed a clearly stronger antibacterial effect against *E. faecalis* in dentine of calcium hydroxide combined with 2 × chlorhexidine than pure calcium hydroxide. Almyroudi et al. (199) did not detect a difference between pure calcium hydroxide and calcium hydroxide combined with 1% chlorhexidine gel. However, in this study, all combinations were effective in the elimination of *E. faecalis* from dentine, contrary to the study by Sukawat & Srisuwan (197). Lynne et al. (200) used only 24 h incubation both for the *E. faecalis* inoculation and for the disinfecting period, and reported better results with 10% calcium hydroxide alone than when combined with 0.12% chlorhexidine.

Using dentine block model, Gomes et al. (201) studied the effect of prolonged incubation with the medicaments on the antibacterial effect of calcium hydroxide and 2% chlorhexidine, alone and in combination. Calcium hydroxide was ineffective for the duration of the experimental period of 30 days, while both chlorhexidine alone and combined with calcium hydroxide killed *E. faecalis* rapidly. However, both the combination and chlorhexidine alone started to lose their antibacterial effect after 2 and 15 days, respectively (201).

When calcium hydroxide combinations have been tested for their antibacterial effect against *E. faecalis* using the agar diffusion test, no clear differences between pure calcium hydroxide and various combinations have been detected (202, 203). Thus it is obvious that results obtained in agar diffusion tests do not reflect the antibacterial effect of the medicaments in infected dentine. Rather, they compare the ability of materials to diffuse through agar. Poorly diffusing material will have very small zones of inhibition, even if they are potent antimicrobials.

Iodine compounds are widely used antimicrobial agents for disinfection of skin and hard surfaces (204). In dentistry, iodine compounds have been used in disinfecting dentures and soft tissues, while in endodontics they are used to disinfect both surfaces prior to access, and as intracanal medicaments. Iodine potassium iodide (IKI) is more effective than calcium hydroxide against a variety of microorganisms found in root canals. However, its allergenic potential is considered a disadvantage (175, 205–208). The effect of IKI against *E. faecalis* and *E. faecium* has been shown *in vitro* in inoculated dentine. The antibacterial effect of 2%/4% IKI has been shown to be better than that of calcium hydroxide, NaOCl and chlorhexidine (177, 178).

Inactivation of endodontic medicaments

One of the claimed advantages of calcium hydroxide is its supposed long-lasting effect, allowing its use as intracanal dressing up to several months. This assumption is based on the stability and other physical properties of calcium hydroxide paste, and the slow release of calcium and hydroxyl ions. Similarly, it has been assumed that several other root canal medicaments, used as vapors or in liquid form, may rapidly lose some or all of their antimicrobial activity in the root canal (209, 210). Therefore, it is surprising how little is known about the activity and inactivation of medicaments in the root canal. Obviously, difficulties in conducting experiments *in vivo* and the lack of suitable *in vitro* models are the main reasons for the lack of data in this area. Haapasalo & Ørstavik (176) developed a root dentine block model, where the block was inoculated with bacteria and subsequently disinfected by medicaments applied into the main canal. This model has since been used in many studies; it has clearly demonstrated a discrepancy between results obtained in test tubes and in inoculated dentine, suggesting that there are factors/mechanisms in the root dentine that protect the bacteria against the antibacterial effect of the medicaments. However, the dentine block model is technically demanding and time consuming, and allows the study of only those microbes that colonize the dentinal tubules in a reasonable time. Moreover, it is not optimally suited for the study of inactivation of medicaments. Therefore, a new method was developed that utilizes root dentine from extracted teeth crushed into powder with a small particle size (211). Experi-

ments using the dentine powder model and *E. faecalis* as the test organisms have shown that the dentine powder totally inhibited the antibacterial activity of saturated calcium hydroxide solution. Likewise, 0.2%/0.4% iodine potassium iodide lost its activity against *E. faecalis* in the presence of dentine powder (211). The activity of 0.05% chlorhexidine and 1% NaOCl was delayed, but not totally abolished. Further studies showed variable inhibition of different root canal medicaments by the dentine powder, bovine serum albumin, hydroxylapatite and dentine matrix (collagen). Interestingly, heat-killed microbial cells of *E. faecalis* and *C. albicans* were also potent inhibitors of the root canal disinfectants (212, 213).

The new results related to the inhibition of the antibacterial activity of locally used irrigating solutions and disinfectants by substances present in the necrotic root canal greatly facilitate our understanding of *in vivo* effects of endodontic medicaments. As the studies also have shown that various medicaments are inhibited/inactivated differently by the substances, it is possible that in the future a cocktail of local disinfectants will be used to optimize canal disinfection.

Susceptibility of oral enterococci to antibiotics

Antibiotics are not considered to be a routine part of endodontic treatment. Only in cases with general indications for antibiotic use should they be prescribed (214). In addition, the effect of systemic antibiotics against bacteria residing in the root canal system is supposed to be poor. Moreover, *E. faecalis* has not been reported in severe spreading infections from the root canal. Therefore in endodontics, from a clinical point of view, the antibiotic susceptibility of *E. faecalis* may not be of major importance. There are no clinical studies showing the effectiveness of systemic antibiotics in endodontic enterococcal infections. Nevertheless, as enterococci are a common cause of bacterial endocarditis, the antibiotic susceptibility of endodontic enterococci is of interest. Enterococci are naturally resistant to nitroimidazoles, which are active only against obligately anaerobic bacteria. The effect of clindamycin against enterococci is also known to be poor (5, 215). Very little is published about the susceptibility of oral or endodontic enterococci to antibiotics, and the number of studied strains has been relatively small (145, 216). So far, no vancomycin-resistant strains have been

reported in endodontic infections, whereas results with other antibiotics, such as ampicillin and benzylpenicillin, have been variable. Molander & Dahlén (217) reported a good anti-enterococcal effect *in vivo* by a locally used mixture of calcium hydroxide and erythromycin, as *Enterococcus* sp. was eliminated from 26 of 27 root canals during the treatment. However, the combination medicament had a rather poor effect against other bacteria present in the canals.

Eradication of *E. faecalis* from infected root canals in vivo

Very little clinical data are available about the effect of locally used root canal disinfectants on enterococci. However, it seems that *E. faecalis* is the most resistant bacterial species to chemomechanical preparation, including instrumentation and irrigation with EDTA and NaOCl (168, 169, 171, 218), and its relative proportion in the post-debridement flora is higher than initially. Increased numbers of some other microbial species usually not present in primary apical periodontitis, such as yeast and Gram-negative enteric rods, have also been reported in teeth with post-treatment apical periodontitis (167, 168, 171, 219, 220). Peciulienė et al. (171) showed that the routine chemomechanical preparation with 5.25% NaOCl did not predictably eliminate *E. faecalis* from the root canal. However, 5-min irrigation with 2%/4% IKI after the chemomechanical preparation eradicated *E. faecalis* in four of five teeth (171). Molander et al. (221) demonstrated that *E. faecalis* could survive in the prepared root canal even after extended periods of dressing with iodine potassium iodide and calcium hydroxide.

Although there is a well-documented resistance against several medicaments, the occurrence of *E. faecalis* in the infected root canal does not seem to be associated with the previous use of certain medicaments or filling materials such as calcium hydroxide (167, 171). The ecological environment in the filled root canal is demanding, and only those microbes that can adjust to such conditions have the possibility of establishing themselves in the canal.

Concluding remarks

E. faecalis is part of the human normal flora and an important pathogen in opportunistic infections in

humans. It is ecologically tolerant and has the ability to survive harsh conditions. In endodontics, *E. faecalis* is rarely present in primary apical periodontitis, but it is the dominant microorganism in root-filled teeth presenting with post-treatment apical periodontitis. It is often isolated from the root canal in pure culture, but it can also be found together with some other bacteria or yeasts. When in mixed infections, *E. faecalis* typically is the dominant isolate. While there is no doubt about the pathogenicity of *E. faecalis* in endodontic infections, it seems to be rarely associated with acute infections and flare-ups. Eradication of *E. faecalis* from the root canal remains a challenge, while chlorhexidine and combinations of disinfectants show some promise. Very few, if any, studies have focused on the impact of *E. faecalis* on the outcome of endodontic treatment; therefore, the answer to this question remains unclear.

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